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OPTIMAL CONDITIONS FOR THE PRODUCTION OF COMMERCIAL ENZYME BY *PENICILIUM LILACINUM* BY CULTURING ON AGRO INDUSTRIAL WASTE

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ABSTRACT

Date syrup is a liquid waste of date processing industries. It is rich in reducing and non reducing sugars and may be used as a fermentation substrate. Cotton stalk is rich in cellulose and hemi cellulose. Pretreated (Physical and chemical) cotton stalk is also used as a carbon source for the production and optimization of industrial enzyme, Invertase (β-fructofuranosidase, E.C.3.2.1.26). Different nitrogen sources (corn-steep, casein, peptone potassium nitrate etc.) different pH (4-9) and temperatures (20-55) have been used. In the present study an indigenous fungus *Penicilium Lilacinum* is used for enzyme production. It was found that invertase production was maximum after 72 hours (on 0.6 *N* H_2SO_4 treated cotton stalk) and 96 hours (on 5% date syrup), production is enhanced by peptone as a nitrogen source at pH 8 and at a temperature of 40 ^oC

Key words: Enzyme, invertase, fermentation, *Penicilium lilacinum*

INTRODUCTION

Various fungi such as *Aspergillus oryzae* (Kenji *et al*., 2010),*Paenibacillus campinasensis* (Chun-Han Ko *et al*., 2010),*Bacillus pumilus* HY1 (Kye *et al*., 2009), *Phanerocheate chrysosporium* (Alam *et al*.,2009), *Citrobacter freundii* MTCC 2424 (Meenakshi *et al*., 2009), *Streptomyces gulbargensis* (Dastager *et al*., 2009), *Aspergillus sojae* (Canan *et al*., 2008), *Aspergillus ficuum* AF-98, (Fengxia *et al*., 2008), *Aspergillus ochraceus* (Luis *et al*., 2007) have been reported for the production of different enzymes.

Microorganisms, such as *Saccharomyces cerevisiae* (Herwig *et al*., 2001, Uroš *et al*., 2010) are used as sources of invertases with biotechnological potential. Filamentous fungi, However, display higher potential for the production of invertases of industrial interest, for instance, those produced by *Rhodotorula glutinis* (Rubio *et al*., 2002), *Aspergillus fumigatus* (Gill *et al*., 2006), *Aspergillus ochraceus* (Guimarães *et al*., 2001) and *Aspergillus niger* (Ashokumar *et al*., 2001). Specific interest has been focused on agro industrial and agriculture waste (date syrup, cotton stalks etc.) because no work is reported about these as a carbon source for the production of industrial enzymes by *Penicilium lilacinum.* In the present work the secretion of invertase by *Penicilium lilacinum* in submerged culture is reported because no work is done on this fungus for the production of invertase.

MATERIALS AND METHODS

STRAIN

Penicillium lilacinum was obtained from the garden of Dr.M.A. Kazi, Institute of Chemistry and the culture was maintained on agar slant, containing (g/L) dextrose 20; peptone 10; agar 20 and distilled water. The ingredients were thoroughly mixed and kept in culture tubes and sterilized at 1.5 kg/cm^2 (Dahot, 1986) for 20 minutes. The sterilized slants were inoculated with *Penicillium lilacinum* and incubated at 27 ^oC to obtain proper growth.

CULTURE MEDIUM

The method for the preparation of culture medium was of Burrell *et al*., (1966). The pH of the culture medium was adjusted to 6.0

PREPARATION OF SPORE SUSPENSION

10.0mL of sterilized water was added to the culture of *Penicillium lilacinum* on agar slant and the surface was rubbed gently with sterilized wire loop (Dahot and Memon, 1987). 1mL of spores suspension contains around 5.8 X 10^7 conidia/mL (Mamma *et al*., 2008).

HYDROLYSIS OF AGRICULTURE WASTE

Cotton stalks were grinded to convert it into powdered form. Ten gram of it was hydrolyzed with 800 mL of 0.3 *N* and 0.6 *N* H_2SO_4 for two hours on flame, maintaining the level of slurry constant. The digested slurry was autoclaved for 30 minutes at 105 kg/cm^2.The slurry was filtered through Whatman No.1 filter paper after cooling at room temperature .The filtrate of solubilised agricultural waste was incorporated into mineral medium as a carbon source. The loss in weight was determined after drying at 105 ^oC to constant weight (Dahot and Abro, 1994). Date syrup was used directly before autoclaved.

CULTIVATION CONDITIONS

100mL of culture media supplied with cotton stalk soluble filtrate or date syrup were taken in conical flasks plugged with cotton wool and autoclaved at 1.5 kg/cm^2 for 20 minutes. The sterilized media cooled at room temperature were inoculated with 1mL of *Penicillium lilacinum* spores containing 5.8 X 10^7 conidia/mL. Pure sugars were sterilized separately and added asceptically. These flasks were incubated in an orbital cooled shaking incubator (Gallenkamp) at 27±2^oC adjusted at 200 rev/minutes. The culture broth was separated from mycelium after an interval of 24 hours incubation period by filtration through What man No.1 filter paper. The enzyme activity, final pH and other parameters were checked from culture broth (Dahot *et al*., 1993)

DETERMINATION OF MYCELIAL BIOMASS

The quantity of the mycelium was noted after washing with distilled water and drying at 110^oC in a hot oven until a constant weight was obtained (Dahot *et al*., 1993).

DETERMINATION OF FINAL pH VALUES

The final pH of culture broth was determined by using WPA pH meter (Dahot *et al*., 1993).

DETERMINATION OF TOTAL SUGAR

The total sugar of digested agriculture waste, date syrup and culture broth was determined by phenol sulfuric acid method (Dubois *et al*., 1956) with glucose as a standard.

DETERMINATION OF REDUCING SUGAR

Reducing sugar in digested agriculture waste, date syrup and culture broth were determined by DNS method (Miller, 1959) with glucose as a standard.

DETERMINATION OF ENZYME ACTIVITY

Invertase activity was determined was determined in 2mL containing 1mM sucrose,20mM acetate buffer pH 4.6,0.1mL of culture broth incubated at 37^oC.After 30 minutes aliquots of 0.2 mL assay mixture were withdrawn and 0.5 mL of 3,5-Dinitrosalicylic acid was added. The solutions were boiled for 10 minutes in a boiling water bath. The color developed was measured at 540 nm according to Bernfeld method. The results of invertase activity were reported /mL of culture broth. One unit of enzyme activity was equal to one mg reducing sugar per minute under the condition of the assay (Bernfeld, 1955).

RESULTS AND DISCUSSION

An agricultural waste hydrolyzed with sulfuric acid produces a variety of sugars and their degradation products. The hydrolysis of cotton stalks to fermentable sugars was carried out by 0.3*N* and 0.6*N* H_2SO_4 and the percentage solubility, total sugars and reducing sugars are shown in Table-1 and 2, respectively while total and reducing sugar in the date syrup in the Table-1A.

The effects of different carbon sources (date syrup and cotton stalk) on the production of invertase by *Penicillium lilacinum* were studied and result are presented in table-3, 4, 5, and 6. It is clear from these tables that time period and maximum quantity of invertase varies with carbon source. It is observed that pH of the medium increases with increasing incubation time period while total sugar, reducing sugars decrease. It was found that invertase production was maximum after 72 hours (on 0.6 *N* H_2SO_4 treated cotton stalks, Table-4) and 96 hours (on

5% date syrup, Table-6).The maximum invertase activity was also obtained from *Aspergillus ochraceus* in Khana medium supplemented by sugar cane bagasse at 40^{o}C for 96 hours. (Luis *et al*., *2007).* But use of date syrup and cotton stalk for the production of invertase is not reported in the literature.

Nitrogen sources and their concentrations have major effect on enzyme yield because sucrose metabolism shows a specific physiological response to the presence of nitrogen source (Silveira *et al*., 2000). Various nitrogen sources were used to enhance the enzyme production and peptone is found to be the best (Table-7). The reason for high enzyme yield might be positive influence of urease and invertase on each other's production because various extracellular enzymes produced by fungus enhance each other's secretion into the culture medium (Egorov *et al*., 2000).

A temperature of 40^{o}C was the optimum temperature (Table-8) which is lower than that for other fungi (Ashokumar *et al*.2001, Dahot 1986, Diomi *et al*. 2008, Guimarães *et al*. 2007, Herwig *et al*. 2001, Luis *et al*. 2007, Rubio *et al*. 2002, Uroš *et al*. 2010).

A wide range of pH (4.0-9.0) was studied and it is found that pH of 8.0 is the best for optimum enzyme production (table-9).however optimal pHs from 2.6 to 6.5 have been reported for invertases from different yeast and fungi (Rubio *et al*.2002, Chaudhuri and Maheshwari 1996, Bhatti *et al*.2006, L'Hocine *et al*.2000, Quiroga *et al*.1995 Chavez *et al*.1997).

Table 1. Effect of 0.3*N* H_2SO_4 on hydrolysis of cotton stalk, percentages of solubility, total sugar and reducing sugar yield.

Parameters	Cotton stalk
Initial mass	10.00
Loss of mass	1.80g
Total sugar in filtrate	175µg/mL
Reducing sugar in filtrate	132µg /mL

Table 1A.

Parameters	Date syrup
Total sugar in filtrate	357 µg/mL
Reducing sugar in filtrate	279 µg /mL

Table 2. Effect of 0.6*N* H_2SO_4 on hydrolysis of cotton stalk, percentages of solubility, total sugar and reducing sugar yield.

Parameters	Cotton stalk
Total sugar in filtrate	253µg/ml
Reducing sugar in filtrate	186µg/mL
Loss of mass	2.78g
Initial mass	10g
Final mass	7.22g
% of hydrolysis	27.8%

Table 3. Effect of 0.3*N* H_2SO_4 pretreated cotton stalk at 27°C and initial pH of 6.0.

Time period in hours	pH	Biomass in (g)	Total sugar OD+0.0225/3.6 µg/mL	Reducing sugar OD+0.05/3.66 µg/mL	Invertase activity units/mL
24	5.65	0.09	1.21	0.61	0.27
48	5.79	0.12	1.02	0.54	0.34
72	5.85	0.16	0.97	0.39	0.45
96	5.92	0.23	0.82	0.24	0.49
120	*6.02*	*0.28*	*0.57*	*0.13*	*0.52*
144	6.12	0.35	0.19	0.02	0.34
168	6.28	0.43	0.08	0.01	0.23
192	6.41	0.49	0.01	0.00	0.12
216	6.42	0.52	0.00	0.00	0.08
240	6.50	0.51	0.00	0.00	0.03

Table 4. Effect of 0.6*N* H_2SO_4 pretreated cotton stalk at 27°C and initial pH of 6.0.

Time period in hours	pH	Biomass (g)	Total sugar OD+0.0225/3.6 µg/mL	Reducing sugar OD+0.05/3.66 µg/mL	Invertase activity units/mL
24	5.41	0.07	1.13	0.58	0.21
48	55.32	0.05	1.02	0.51	0.34
72	*5.47*	*0.11*	*0.98*	*0.42*	*0.51*
96	5.54	0.25	0.85	0.39	0.32
120	5.57	0.32	0.37	0.21	0.14
144	5.69	0.47	0.19	0.13	0.03
168	5.74	0.53	0.12	0.09	0.04
192	5.93	0.59	0.04	0.01	0.03
216	5.97	0.62	0.01	0.00	0.01

Table 5. Effect of 2.5% date syrup at 27°C and initial pH of 6.0.

Time period in hours	pH	Biomass (g)	Total sugar OD+0.0225/3.6 µg/mL	Reducing sugar OD+0.05/3.66 µg/mL	Invertase activity units/mL
24	5.92	0.29	2.34	1.42	0.61
48	5.99	0.37	2.52	1.26	0.67
72	6.12	0.35	1.37	1.12	0.78
96	*6.21*	*0.41*	*1.07*	*0.76*	*0.82*
120	6.31	0.49	0.87	0.41	0.62
144	6.39	0.53	0.42	0.21	0.43
168	6.52	0.58	0.21	0.09	0.25
192	6.59	0.68	0.15	0.02	0.09
216	6.71	0.71	0.09	0.01	0.02
240	6.77	0.92	0.02	0.00	0.01

Table 6. Effect of 5% date syrup at 27°C and initial pH of 6.0.

Time period in hours	pH	Biomass (g)	Total sugar OD+0.0225/3.6 µg/mL	Reducing sugar OD+0.05/3.66 µg/mL	Invertase activity units/mL
24	5.97	0.42	4.42	3.32	1.17
48	6.18	0.51	4.37	3.11	1.25
72	6.22	0.57	3.85	2.59	1.51
96	*6.31*	*0.62*	*2.85*	*1.90*	*1.67*
120	6.47	0.87	2.17	1.12	1.24
144	6.43	1.15	1.25	0.68	1.02
168	6.49	1.13	0.97	0.34	0.76
192	6.57	1.19	0.76	0.19	0.39
216	6.65	1.21	0.57	0.06	0.06
240	6.75	1.25	0.58	0.08	0.01

Table 7. Effect of nitrogen sources with 5% date syrup at 96 hour, 27°C and initial pH of 6.0.

Nitrogen source	Amount in grams	enzyme activity in unit/mL
Corn steep	0.25	2.421
	0.5	2.542
Casein	0.25	2.321
	0.5	2.259
Peptone	*0.25*	*2.672*
	0.5	2.521
Potassium nitrate	0.25	2.215
	0.5	2.125
Albumin	0.25	2.273
	0.5	2.349
Ammonium sulphate	0.25	2.342
	0.5	2.526
Urea	0.25	2.124
	0.5	2.215
Yeast extract	0.25	2.185
	0.5	2.210

Table 8. Effect of temperature with Nitrogen source (peptone) with 5% date syrup at 96 hour and at initial pH of 6.0.

Temperature in Celsius	Enzyme activity in units/mL
20	2.154
25	2.845
30	2.920
35	3.152
40	*3.825*
45	3.012
50	2.134
55	1.672

Table 9. Effect of pH with Nitrogen source (peptone) with 5 % dates syrup at 96 hour and At initial pH of 6.0.

pH	Enzyme activity in units/mL
4.0	1.752
4.5	1.821
5.0	2.125
5.5	2.225
6.0	2.321
6.5	2.359
7.0	2.598
7.5	2.890
8.0	*2.920*
8.5	2.125
9.0	1.821

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